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# Oxygen-18 and carbon-13 isotopes in eCO<sub>2</sub> and erythrocytes carbonic anhydrase activity of Finnish prediabetic population

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Supplementary material for this article is available [online](#)

## Abstract

Complex human physiological processes create the stable isotopic composition of exhaled carbon dioxide (eCO<sub>2</sub>), measurable with noninvasive breath tests. Recently, isotope-selective breath tests utilizing natural fluctuation in <sup>18</sup>O/<sup>16</sup>O isotope ratio in eCO<sub>2</sub> have been proposed for screening prediabetic (PD) individuals. It has been suggested that <sup>18</sup>O/<sup>16</sup>O fractionation patterns reflect shifts in the activity of carbonic anhydrase (CA), an enzyme involved in the metabolic changes in the PD state. To evaluate the applicability of the breath sampling method in Finnish PD individuals, breath delta values (BDVs, ‰) of <sup>18</sup>O/<sup>16</sup>O ( $\delta^{18}\text{O}$ ) were monitored for 120 min in real-time with a high-precision optical isotope ratio spectrometer, both in the fasting state and during a 2 h oral glucose tolerance test (2 h OGTT) with non-labeled glucose. In addition, the BDV of <sup>13</sup>C/<sup>12</sup>C ( $\delta^{13}\text{C}$ ) was measured, and total erythrocyte CA activity was determined.  $\delta^{18}\text{O}$  and CA did not demonstrate any statistically significant differences between PD and non-diabetic control (NDC) participants. Instead,  $\delta^{13}\text{C}$  was significantly lower in PD patients in comparison to NDCs in the fasting state and at time points 90 and 120 min of the 2 h OGTT, thus indicating slightly better potential in identifying Finnish PD individuals. However, overlapping values were measured in PD participants and NDCs, and therefore,  $\delta^{13}\text{C}$  cannot be applied as a sole measure in screening prediabetes at an individual level. Thus, because the combination of environmental and lifestyle factors and anthropometric parameters has a greater effect on glucose metabolism and CA activity in comparison to the PD state, <sup>18</sup>O/<sup>16</sup>O and <sup>13</sup>C/<sup>12</sup>C fractionations or CA activity did not prove to be reliable biomarkers for impaired glucose tolerance in Finnish subjects.

This study was conducted under the clinicaltrials.gov ID NCT03156478.

## 1. Introduction

The stable isotopic composition of exhaled carbon dioxide (eCO<sub>2</sub>) displays characteristics of the physical process of origin of the molecule. Thus, isotopic recovery via noninvasive breath tests can provide signatures, for example, for many complex human physiological phenomena, such as diseases, medical conditions, energy consumption, and changes in various body functions [1, 2].

The stable isotope breath tests are commonly based on ingestion of a carbon-13 (<sup>13</sup>C)-labeled substrate, which is converted into <sup>13</sup>CO<sub>2</sub> via metabolism and is detected as a change in the baseline <sup>13</sup>C/<sup>12</sup>C ratio in eCO<sub>2</sub>. The most well-known example among isotope-selective breath tests is the <sup>13</sup>C-labeled urea breath test (<sup>13</sup>C-UBT), used to identify *Helicobacter pylori* infection. United States Food and Drug Administration has approved the <sup>13</sup>C-UBT already in the 1990s. Labeled <sup>13</sup>C tracers have also been used to

assess insulin sensitivity [3] and glucose uptake [4] as well as in numerous applications of medicine [5] and nutrition [6]. Along with labeled  $^{13}\text{C}$ , the measurement of oxygen-16 ( $^{16}\text{O}$ ) and oxygen-18 ( $^{18}\text{O}$ ) isotopes in exhaled breath can be exploited in noninvasive studies and diagnostic approaches [7–9].

The latest research lines in the field of isotope-selective breath testing include early sepsis and infection detection [7, 10–12] and prediabetes (PD) and type 2 diabetes (T2D) screening [8]. These applications are based on monitoring the breath delta values (BDVs, ‰) of  $^{18}\text{O}/^{16}\text{O}$  and/or  $^{13}\text{C}/^{12}\text{C}$  in  $\text{eCO}_2$  (i.e.  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$ ) and rely on the natural fluctuation in the isotope ratios during innate or induced metabolic events [2, 9, 11]; thus, no labeling is used. Typically, delta-over-baseline (DOB, ‰) values, i.e. shift in BDV in relation to the baseline, are utilized in diagnostic purposes. BDVs of  $^{18}\text{O}/^{16}\text{O}$  and  $^{13}\text{C}/^{12}\text{C}$  are often calculated in relation to an international Vienna Pee Dee Belminite (VPDB) standard. DOB values (i.e.  $\delta_{\text{DOB}}^{18}\text{O}$  and  $\delta_{\text{DOB}}^{13}\text{C}$ ) are calculated as described:  $\text{DOB} = \text{BDV}_{\text{sample}} - \text{BDV}_{\text{baseline}}$ . Recently, there have been attempts to utilize  $\delta^{13}\text{C}$ , as such, for example in sepsis detection and severity evaluation [10], and in supporting weight-management by monitoring energy metabolism [13].

Based on the recent studies by Ghosh *et al* [8], monitoring of  $^{18}\text{O}/^{16}\text{O}$  isotope ratios during the 2 h oral glucose tolerance test (2 h OGTT) provides a potential non-invasive method for the assessment of glycated hemoglobin (HbA1c) and insulin sensitivity in Indian participants with PD and T2D. Furthermore, they proposed that  $^{18}\text{O}/^{16}\text{O}$  fractionation patterns reflect carbonic anhydrase (CA) activity in PD individuals and T2D patients: fasting CA activity was shown to be reduced in T2D patients in comparison to PD and non-diabetic controls (NDCs), and this phenomenon was suggested to be related to the increased glycosylation of CA in T2D patients [8]. CA enzymes, divided into 7 subgroups and comprising 15 isoforms, are present in various human tissues, and catalyze the conversion of  $\text{CO}_2$  to bicarbonate ( $\text{HCO}_3^-$ ) in blood and tissues, thus participating in the pH regulation of the human body [14]. Ghosh *et al* [8] suggested that while CA also induces the efficient exchange of oxygen isotopes between blood plasma  $\text{H}_2^{18}\text{O}$  and  $^{12}\text{C}^{16}\text{O}_2$  during respiration, higher CA activity results in a higher  $^{18}\text{O}/^{16}\text{O}$  ratio in exhaled breath. That is, a change in CA activity can be seen as a shift in  $\delta^{18}\text{O}$  in  $\text{eCO}_2$ , reflecting the  $^{18}\text{O}/^{16}\text{O}$  ratio in body  $\text{H}_2\text{O}$  and  $\text{CO}_2$ . Changes in CA activity seem to be involved in the altered metabolism in different stages of T2D [15], and thus, isotopic enrichment of  $^{18}\text{O}$  in  $\text{eCO}_2$  could be observed in PD individuals and T2D patients [8].

However, the  $\delta^{18}\text{O}$  in the fasting state and during the 2 h OGTT were not reported by Ghosh *et al* [8], and the  $\delta_{\text{DOB}}^{18}\text{O}$  values were used to express the

$^{18}\text{O}/^{16}\text{O}$  stable isotope ratios. Thus, to evaluate the more universal applicability of the breath sampling method and to study the potential trends in  $^{18}\text{O}/^{16}\text{O}$  isotopic fractionation in the fasting state or during the 2 h OGTT,  $\delta^{18}\text{O}$  was monitored in Finnish PD individuals for 120 min in real-time with a compact VTT IsoMed Breath  $\text{CO}_2$  isotope analyzer [16]. Furthermore, CA activity and HbA1c concentrations were measured to study the correlations between fasting  $\delta^{18}\text{O}$  and HbA1c levels and CA activity. In addition, as breath  $^{13}\text{C}/^{12}\text{C}$  isotope ratio has been established as a valuable biomarker in the assessment of insulin resistance in tracer approaches [17, 18], breath  $\delta^{13}\text{C}$  was measured simultaneously to investigate whether this approach would provide new means for the evaluation of T2D prognosis. The anthropometric measures of the participants were also surveyed against  $\delta^{18}\text{O}$ ,  $\delta^{13}\text{C}$ , and CA activity. Furthermore, several clinically relevant blood values were screened in the fasting state and during the 2 h OGTT for potential interactions between  $\text{CO}_2$  isotopic fractionations and the prediabetic (PD) state.

## 2. Materials and methods

### 2.1. Study subjects

Two groups of participants, consisting of men and women aged 40–65, were recruited for the study. Altogether, 30 PD individuals were recruited among the participants in the ongoing StopDia study [19]. The StopDia study was approved by the Research Ethics Committee of Hospital District of Northern Savo (statement number 467/2016). The inclusion criteria for the PD participants were: BMI 25–35  $\text{kg m}^{-2}$ , fasting plasma glucose 5.6–6.9  $\text{mmol l}^{-1}$ , and 2 h OGTT plasma glucose 7.8–11.0  $\text{mmol l}^{-1}$ . The 19 NDC participants were recruited via e-mail or intranet notification to the staff of the University of Eastern Finland and Kuopio University Hospital. The extension of the study group with NDC individuals was approved by the Research Ethics Committee of Hospital District of Northern Savo (statement number 542/2017). Written informed consent to participate in the study and the use of data from national health care registries were obtained from all participants. The study was conducted according to the Responsible Conduct of Research by the Finnish Advisory Board on Research Integrity and the Declaration of Helsinki.

The participants arrived in the laboratory in the morning after 12 h fasting. At the beginning of the visit, all participants were weighed, and waist circumference and systolic and diastolic blood pressure were measured. The participants were interviewed for their health status and recent travel history. The participants were randomly selected to go through a short test routine, or a long test routine involving the 2 h OGTT: 10 of the 19 NDC participants and 15 of the 30 PD participants continued to the 2 h OGTT.

## 2.2. Breath sampling

For all participants, a maximum 5 min breath sampling was performed with nasal cannula to determine the fasting state  $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$ . The isotopes were measured with a high-precision optical isotope ratio spectrometer (OIRS) developed by VTT Technical Research Centre of Finland [16]. The spectrometer is based on a direct absorption approach using a tunable mid-infrared Interband Cascade Laser. The molecular absorption frequencies of carbon dioxide isotopologues are slightly shifted due to mass difference. This allows for the selective detection of the three most abundant isotopes of  $\text{CO}_2$  in a compact device. Water was removed from the sample using Silica gel capsules, prior introduction to the measurement cell of the spectrometer. The sample gas was actively compared to an isotope standard with a known isotope ratio, previously analyzed by the Max-Planck Institute for Biochemistry [20]. The analysis routine was validated by using gas standards of different mixtures. The precision of the DOB measurements is verified to be within 0.1 ‰ and the absolute accuracy of the BDV within 0.3 ‰ (1 sigma standard deviation) compared to the VPDB- $\text{CO}_2$  scales for  $\delta_{\text{DOB}}^{18}\text{O}$  and  $\delta_{\text{DOB}}^{13}\text{C}$ .

## 2.3. Blood sampling

The NDC participants were subjected to screening blood tests, including blood count, serum thyroid-stimulating hormone, creatinine, alanine aminotransferase, and alkaline phosphatase. The blood sample used for screening tests was collected concurrently with the fasting blood sample; the PD participants had been previously screened during the Stop-Dia study.

The screening blood samples and/or fasting blood samples were collected immediately after breath sampling. The fasting blood sample was analyzed for plasma glucose, serum insulin, HbA1c, blood hemoglobin, high-sensitivity C-reactive protein, and erythrocyte hemoglobin concentrations.

Blood samples were analyzed by the methodology currently in routine use at the Institute of Public Health and Clinical Nutrition and Clinical Research Unit at the University of Eastern Finland and the Clinical Chemistry Laboratory at the Kuopio University Hospital. Blood glucose levels were analyzed using the Konelab 20XTi Clinical Chemistry Analyser (Thermo Fisher Scientific, Waltham, MS, U.S.) and an enzymatic photometric (glucose hexokinase) method, and serum insulin levels were analyzed with a chemiluminescent immunoassay. HbA1c (%) was determined by high performance liquid chromatography by utilizing an HbA1c analyzer. High-sensitivity C-reactive protein concentrations were determined by nephelometry (Siemens, Eschborn, Germany).

## 2.4. CA activity

Total erythrocyte CA activity was determined according to Ghosh *et al* [8] from fasting and 2 h OGTT blood samples. In brief, the procedure was as follows: erythrocytes were isolated from fresh EDTA plasma samples by centrifugation. After lysing the erythrocytes, the resulting hemolysate was used for the CA activity measurement. The CA activity was determined spectrophotometrically from the hydrolysis rate of *p*-nitrophenyl acetate to *p*-nitrophenol, monitoring the increase in absorbance at 348 nm. As there are other esterases present in the hemolysate, reactions were executed in the presence of a specific inhibitor of CA, acetazolamide. The total CA activity was calculated from the difference in change of optical densities in the presence and absence of acetazolamide.

## 2.5. 2 h OGTT

For all participants going through the long test routine with 2 h OGTT, after collection of the fasting blood sample, a beverage containing 75 g of non-labeled glucose was given; the fasting blood sample served also as the pre-glucose-load blood sample of the 2 h OGTT. Blood samples were collected at 30, 60, 90 and 120 min after the bolus of glucose and were analyzed for plasma glucose and serum insulin levels as well as CA activity. The isotope measurements were conducted in real-time throughout the 2 h OGTT, using a nasal cannula. For each measurement, the isotope reading was averaged around 5 min to reduce the standard deviation of the measurement, but also to average potential short-term variations of the isotopes in patient breath.

## 2.6. Statistical analyses

The anthropometric measures and breath test values were statistically tested with the SPSS software, Version 27 (IBM SPSS Statistics for Windows, IBM, Armonk, New York, U.S.). Analysis of variance was used for normally distributed variables. Kruskal–Wallis test was used for variables with skewed distributions and for pairwise comparisons of  $\delta_{\text{DOB}}^{18}\text{O}$ ,  $\delta_{\text{DOB}}^{13}\text{C}$ , and  $\Delta\text{CA}$  at each time point. Correlations among variables were analyzed using Pearson's coefficients for correlation. The *p*-value threshold for statistical significance was set at 0.05.

## 3. Results

Anthropometrics, fasting blood glucose, fasting serum insulin, HbA1c, isotopic fractionations and CA activity in the study groups are presented in table 1.

For PD participants, the mean changes in CA activity the ( $\Delta\text{CA}$ ;  $\text{U min}^{-1} \text{ ml}^{-1}$ ) increased during the whole 120 min of 2 h OGTT (figure 1; table S1 which is available online at [stacks.iop.org/JBR/15/021001/mmedia](https://stacks.iop.org/JBR/15/021001/mmedia)). The mean  $\delta_{\text{DOB}}^{18}\text{O}$  (‰) showed fluctuation first increasing (30 min), then

**Table 1.** The anthropometric and diagnostic blood values, CA activity levels, and breath delta values ( $\delta^{18}\text{O}$ ,  $\delta^{13}\text{C}$ ) ( $\pm$  standard deviation) in NDC subjects ( $n = 19$ ) and in PD participants ( $n = 30$ ). CA activity,  $\delta^{18}\text{O}$ , and  $\delta^{13}\text{C}$  were monitored in 30 min intervals for NDC ( $n = 10$ ) and PD ( $n = 15$ ) subjects participating the long test routine with 2 h OGTT.

	NDC	PD	sig.
Sex (male/female)	7/12	10/20	
Age (years)	52.9 $\pm$ 7.9	59.1 $\pm$ 8.2	0.012 <sup>d</sup>
Weight (kg)	75.5 $\pm$ 16.4	85.7 $\pm$ 14.3	0.025 <sup>d</sup>
BMI (kg m <sup>-2</sup> )	25.5 $\pm$ 3.7	30.4 $\pm$ 3.6	0.000 <sup>d</sup>
Waist (cm)	87.3 $\pm$ 3.0	100.5 $\pm$ 10.5	0.000 <sup>d</sup>
Waist-to-height ratio	0.5 $\pm$ 0.1	0.6 $\pm$ 0.1	0.000 <sup>d</sup>
Fasting plasma glucose (mmol l <sup>-1</sup> )	5.5 $\pm$ 0.4	6.1 $\pm$ 0.6	0.000 <sup>d</sup>
Fasting plasma insulin (mU l <sup>-1</sup> )	7.0 $\pm$ 4.9	15.0 $\pm$ 9.9	0.001 <sup>d</sup>
HbA1c (mmol mol <sup>-1</sup> )	33.9 $\pm$ 2.3	38.0 $\pm$ 1.8	0.000 <sup>d</sup>
Fasting CA activity (U min <sup>-1</sup> ml <sup>-1</sup> )	5.2 $\pm$ 0.9	5.0 $\pm$ 0.8	0.412
Fasting $\delta^{18}\text{O}$ (‰)	-5.6 $\pm$ 1.0 <sup>a</sup>	-6.0 $\pm$ 1.3	0.244
Fasting $\delta^{13}\text{C}$ (‰)	-27.4 $\pm$ 0.6 <sup>a</sup>	-27.8 $\pm$ 0.7	0.048 <sup>d</sup>
2 h OGTT	NDC <sup>b</sup>	PD <sup>c</sup>	sig.
Fasting CA activity (U min <sup>-1</sup> ml <sup>-1</sup> )	5.2 $\pm$ 0.9	4.7 $\pm$ 0.9	0.180
CA activity (U min <sup>-1</sup> ml <sup>-1</sup> ) 30 min	5.4 $\pm$ 0.8	4.9 $\pm$ 0.8	0.154
CA activity (U min <sup>-1</sup> ml <sup>-1</sup> ) 60 min	5.2 $\pm$ 0.4	4.9 $\pm$ 0.8	0.372
CA activity (U min <sup>-1</sup> ml <sup>-1</sup> ) 90 min	5.4 $\pm$ 0.9	5.0 $\pm$ 0.7	0.290
CA activity (U min <sup>-1</sup> ml <sup>-1</sup> ) 120 min	5.5 $\pm$ 0.6	5.3 $\pm$ 0.9	0.529
Fasting $\delta^{18}\text{O}$ (‰)	-5.6 $\pm$ 1.0	-6.2 $\pm$ 1.4	0.295
$\delta^{18}\text{O}$ (‰) 30 min	-5.8 $\pm$ 1.0	-6.2 $\pm$ 1.5	0.561
$\delta^{18}\text{O}$ (‰) 60 min	-5.8 $\pm$ 1.0	-6.2 $\pm$ 1.5	0.467
$\delta^{18}\text{O}$ (‰) 90 min	-5.8 $\pm$ 1.1	-6.1 $\pm$ 1.5	0.621
$\delta^{18}\text{O}$ (‰) 120 min	-5.9 $\pm$ 1.1	-6.2 $\pm$ 1.5	0.605
Fasting $\delta^{13}\text{C}$ (‰)	-27.3 $\pm$ 0.8	-27.6 $\pm$ 0.6	0.246
$\delta^{13}\text{C}$ (‰) 30 min	-26.8 $\pm$ 0.7	-27.1 $\pm$ 0.5	0.375
$\delta^{13}\text{C}$ (‰) 60 min	-26.8 $\pm$ 0.7	-26.3 $\pm$ 0.6	0.085
$\delta^{13}\text{C}$ (‰) 90 min	-24.5 $\pm$ 0.8	-25.3 $\pm$ 0.7	0.025 <sup>d</sup>
$\delta^{13}\text{C}$ (‰) 120 min	-24.0 $\pm$ 0.9	-24.7 $\pm$ 0.7	0.032 <sup>d</sup>

<sup>a</sup>  $n = 18$ .

<sup>b</sup>  $n = 10$ .

<sup>c</sup>  $n = 15$ .

<sup>d</sup> Statistically significant (sig.) difference,  $p$ -value < 0.05.

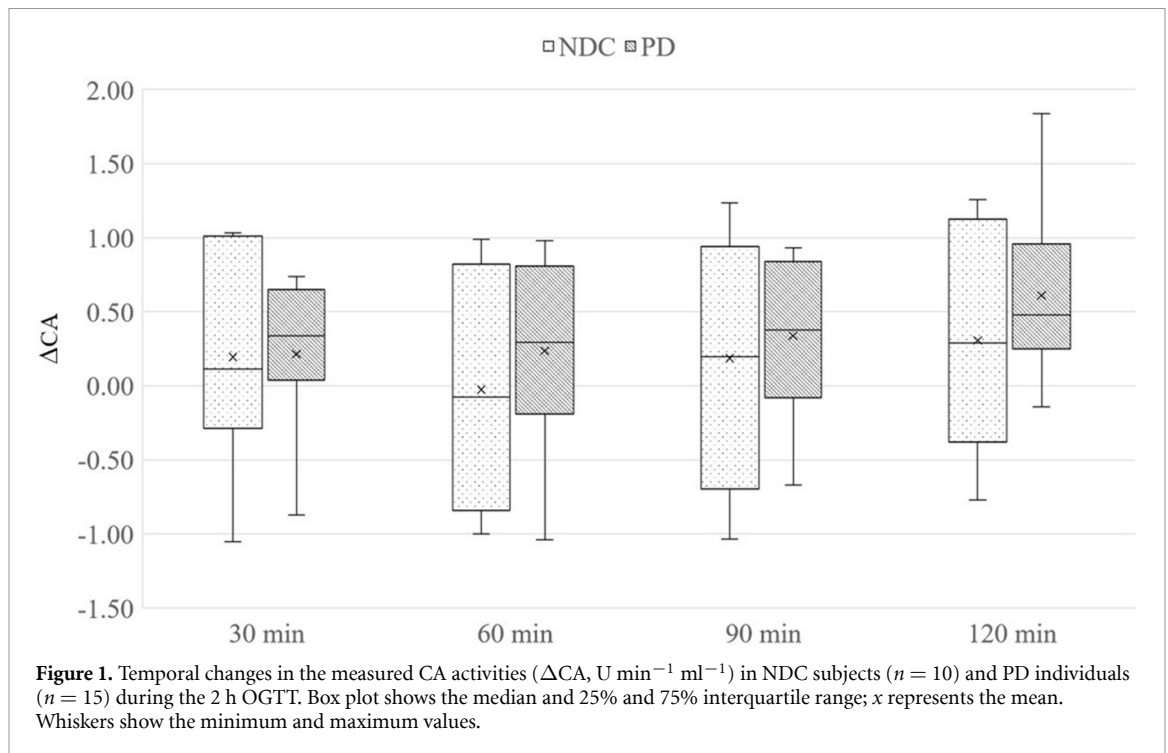
decreasing (60 min), and then increasing again (90 min); and finally, slightly decreasing at time point 120 min (figure 2). For NDC subjects,  $\Delta\text{CA}$  was, on average, increased during the first 30 min of 2 h OGTT, and after that, at time point 60 min,  $\Delta\text{CA}$  was decreased close to the initial value measured at the time point 0 (figure 1). At time points 90 and 120 min,  $\Delta\text{CA}$  was increasing in NDC group. Meanwhile,  $\delta_{\text{DOB}}^{18}\text{O}$  was first decreased (at 30 min), then slightly increased (at 60 min), and again decreased (at 90 and 120 min) (figure 2). At time point 30 min, there was a significant difference ( $p = 0.037$ ) in  $\delta_{\text{DOB}}^{18}\text{O}$  between groups.  $\delta_{\text{DOB}}^{13}\text{C}$  was more steeply increased in NDC group during the 2 h OGTT (figure 3; table S1).

The absolute CA activity measures and  $\delta^{18}\text{O}$  were, on average, slightly lower in PD participants in comparison to NDC group at all time points of measurement (table 1). However, the fluctuations in CA activity levels and in  $\delta^{18}\text{O}$  were minor and showed no statistically significant differences between the two study groups. Fasting  $\delta^{13}\text{C}$  was significantly lower in

PD participants ( $n = 30$ ) in comparison to NDC subjects ( $n = 19$ ) (table 1), and at time points 90 and 120 min of the 2 h OGTT,  $\delta^{13}\text{C}$  was decreased to a significantly lower level in PD versus NDC subjects. Fasting  $\delta^{13}\text{C}$  showed negative correlation with fasting HbA1c level ( $r = -0.306$ ,  $p < 0.05$ ); however, there was no significant correlation between fasting HbA1c level and fasting  $\delta^{18}\text{O}$  or fasting CA activity. Instead, fasting  $\delta^{18}\text{O}$  was found to positively correlate with age ( $r = 0.339$ ,  $p < 0.05$ ), and the fasting state CA activities showed small but significant negative correlation with participant's body mass index (BMI; kg m<sup>-2</sup>) ( $r = -0.286$ ,  $p < 0.05$ ). Furthermore, BMI was significantly higher in PD group (table 1).

Indeed, as expected, higher BMI, waist circumference, waist-to-height ratio, blood insulin and glucose levels, as well as HbA1c values served as moderate indicators of PD status. However, overlapping in the measured values was observed in the participant groups. Other blood sample values showing statistically significant differences between NDC and PD groups were: leucocyte count, erythrocyte





count, hemoglobin, hematocrit, mean corpuscular volume (MCV), high-sensitivity C-reactive protein, and plasma alkaline phosphatase (data not shown). All values were positively interrelated with PD state, except for MCV, which was, on average, higher in NDC subjects.

#### 4. Discussion

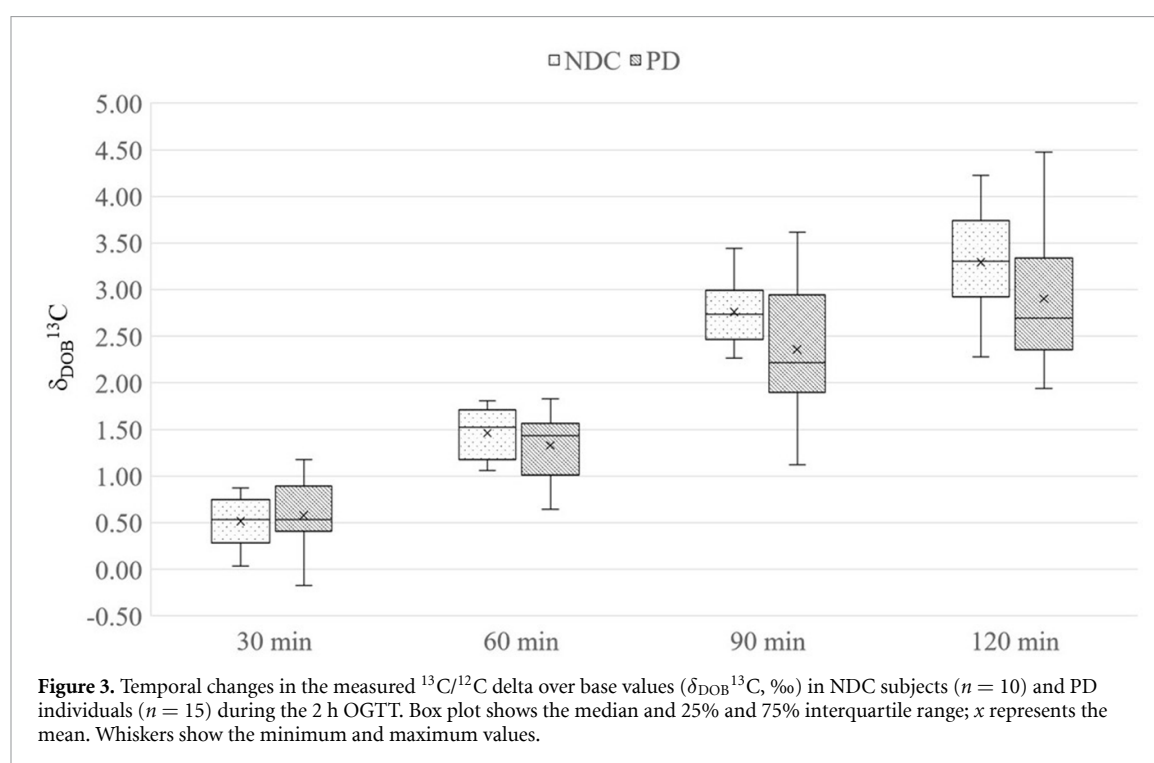
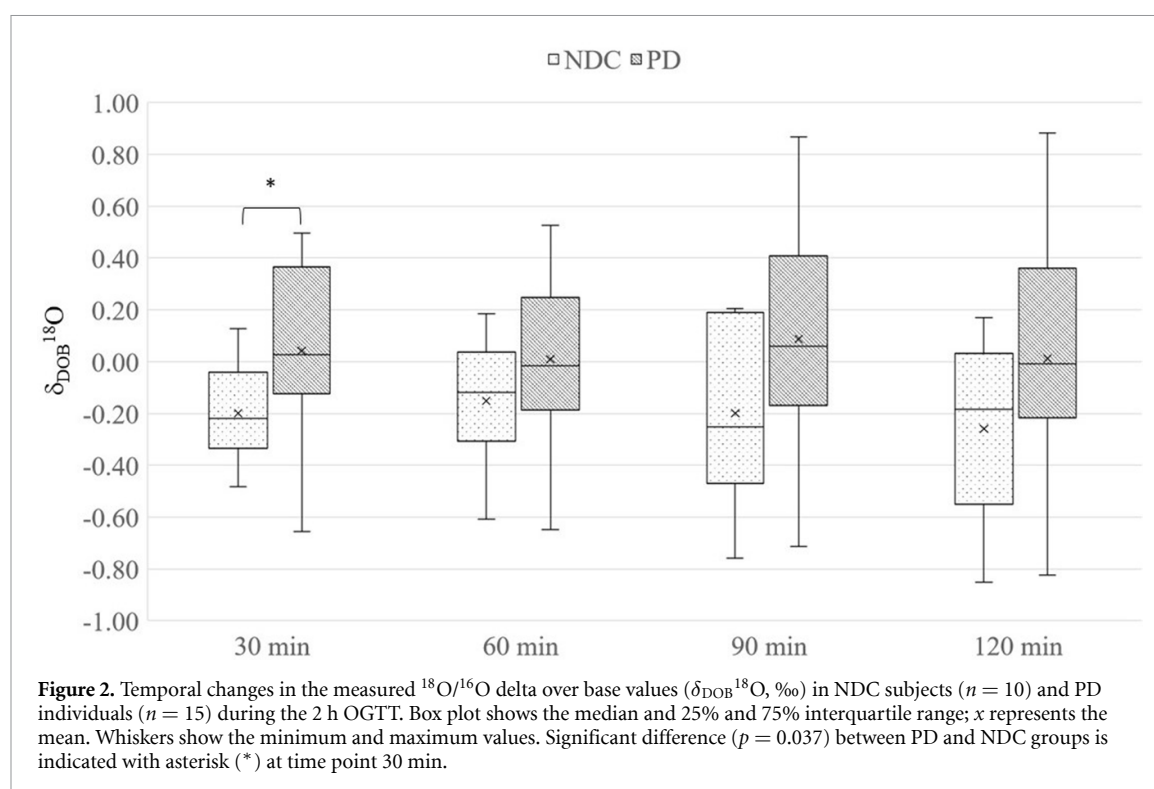
Based on the results of this study, measuring  $\delta^{13}\text{C}$  during 2 h OGTT could support the identification of Finnish PD individuals, as characteristic  $\delta^{13}\text{C}$  in PD and NDC subjects seemed to indicate the differences in insulin response and glucose metabolism between the study groups. The shift in isotopic fractionation of breath  $\text{CO}_2$  after ingesting a bolus of glucose reflects the rate of glucose absorption, insulin response, and glucose uptake in tissues [17]. In previous studies it has also been found that breath  $\delta_{\text{DOB}}^{13}\text{C}$  serves as an indicator of *H. pylori* infection, known to affect glucose metabolism, when unlabeled glucose has been applied in OGTT [7]. Thus, the more pronounced appearance and slower depletion of  $^{13}\text{C}$  in  $\text{eCO}_2$  during the 2 h OGTT indicates that glucose is more effectively metabolized in NDC subjects in comparison to PD participants of our study. Nevertheless, because overlapping values were measured in PD and NDC subjects in our analyses,  $\delta^{13}\text{C}$  cannot be recommended as an only measure in screening prediabetes on an individual level.

The results of the present study indicated that the CA activity steadily increased during the 120 min of the 2 h OGTT in the PD individuals and slightly decreased during the first 60 min of the 2 h OGTT in

the NDC individuals. Thus, our results were in line with the results of Ghosh *et al* [8] who showed that CA activity clearly increased in PD individuals and steeply decreased in NDC individuals during the 2 h OGTT; however, Ghosh *et al* [8] had a single measure at 120 min after the glucose dose, while we had measurements every 30 min during the 2 h OGTT. In contrast to Ghosh *et al* [8], we found no statistically significant differences between the study groups. In addition, in our study  $\Delta CA$  were smaller, and the CA activity increased above the fasting level in the NDC individuals.

For  $\delta_{\text{DOB}}^{18}\text{O}$ , our results showed that in PD participants, on average, there were no notable mean changes, although a minor increase in  $\delta_{\text{DOB}}^{18}\text{O}$  could be observed at time point 90 min; in the case of NDC subjects,  $\delta_{\text{DOB}}^{18}\text{O}$  was slightly decreased within the 120 min trial. Instead, Ghosh *et al* [8] observed that for PD patients, the  $\delta_{\text{DOB}}^{18}\text{O}$  was sharply increased within 120 min of OGTT, and for NDC subjects, the values were clearly decreased; in their study,  $\delta_{\text{DOB}}^{18}\text{O}$  determined at time point 120 was significantly different between PD and NDC groups. In our study, significant difference was observed at time point 30 min.

Importantly, although the trends in the average values for CA activity and  $\delta_{\text{DOB}}^{18}\text{O}$  values in Finnish study subjects were in line with the results of Ghosh *et al* [8], there were large variations in the CA activity and  $^{18}\text{O}/^{16}\text{O}$  fractionation patterns between individuals across the study groups. Thus, these measures were not proved as reliable biomarkers for health status in Finnish subjects. Overall, because the biotic and abiotic factors affecting the stable oxygen isotopic values in mammals are complex, it may be difficult to



compare oxygen isotopic data collected from different study sites [21]. The diet, drinking water, exercise routines, and for example, smoking affect the oxygen isotope fractionation in humans [2]. Drinking water oxygen isotopic composition has major differences between Finland and India [22], and these differences may partially explain the poor applicability of the method in screening Finnish PD individuals. In addition, hormone-mediated responses and other

metabolic differences between study groups may have an effect on the  $^{18}\text{O}$  depletion levels [21].

Based on our results it is also suggested that the ratio can change in response to the age of the study subject, as fasting  $\delta^{18}\text{O}$  was significantly positively correlated with age in the present study. The mean age in the study of Ghosh *et al* [8] was  $36.0 \pm 7.5$  and  $34.6 \pm 6.5$  years ( $p > 0.05$ ) for NDC and PD subjects, respectively, as in the current study, the mean



age was  $53.0 \pm 7.9$  and  $59.2 \pm 8.2$  years ( $p < 0.05$ ) for NDC and PD subjects, respectively. Thus, the differences in living environments, lifestyles, and age-related parameters might be reflected in the differing changes in  $\delta^{18}\text{O}$  during 2 h OGTT observed between PD and NDC groups in this study in comparison to Ghosh *et al* [8]. It should be noted that the measuring accuracy of the OIRS equipment used in our study is comparable to the measuring accuracy of the isotopic  $\text{CO}_2$  integrated cavity output spectrometer utilized by Ghosh *et al* [8].

It has been found that CA activity level is somewhat reduced in young obese PD patients in relation to healthy normal-weight subjects, although this reduction was not statistically significant [23]. Actually, the CA activity was slightly positively correlated with obese subjects' BMI [23]. In our data, the absolute CA activity measures were, on average, slightly lower in PD participants in comparison to NDC group at all time points of measurement, and the fasting state CA activity levels ( $5.2 \pm 0.9$  and  $5.0 \pm 0.8$  for NDC and PD subjects, respectively;  $p = 0.412$ ) were negatively correlated with the subjects' BMI. Malheiro *et al* [23] have suggested that in PD children with BMI higher than  $40 \text{ kg m}^{-2}$ , CA activity levels would be increased in comparison to overweight subjects with lower level of obesity. In our study, the average BMI in PD group was  $30.4 \pm 3.6 \text{ kg m}^{-2}$  while the average BMI in NDC group was  $25.5 \pm 3.7 \text{ kg m}^{-2}$ . Thus, no participant had BMI higher than  $37.9 \text{ kg m}^{-2}$ , and the low-to-moderate level of obesity in the participants in PD group would explain the negative analogue between BMI and CA activity. This possible relationship between low-to-moderate level of obesity and CA activity is also slightly supported when the Finnish and Indian studies are compared: our Finnish subjects had higher BMI, as well as lower fasting CA activity levels in both NDC and PD groups, in comparison to the study groups of Ghosh *et al* [8]. Indeed, the average BMI was  $24.28 \pm 2.8$  and  $24.17 \pm 2.4 \text{ kg m}^{-2}$  ( $p = 0.877$ ) and the average fasting CA activity level was  $9.18 \pm 1.2$  and  $8.98 \pm 1.2 \text{ U min}^{-1} \text{ ml}^{-1}$  ( $p < 0.001$ ), for the Indian NDC and PD subjects, respectively [8]. Moreover, in our study, the mean age was  $52.9 \pm 7.9$  and  $59.1 \pm 8.2$  years for NDC and PD groups, respectively, which may further highlight the negative correlation. It should also be taken into account that measuring total erythrocytic CA activity includes the activities of several CA isozymes; this may mask the variable activity levels of different CA isozymes in PD and NDC subjects and complicate the interpretation of the CA activity results [23].

Biswas and Kumar [24] have observed in their *in vitro* studies that CA activity may increase in short term as a result of enhanced methylglyoxal (MG) levels, often measured in insulin resistant patients. MG synthesis is triggered by high blood sugar levels and further enhanced if the person has developed

insulin resistance [24]. MG is considered as a toxic substance, and it is involved in protein glycation reactions affecting erythrocyte membrane integrity, insulin reactivity, as well as CA activity [24]. As MG is produced via secondary activities of glycolysis, functioning under both genetic and environmental regulation [25], there is a chance that the inconsistent relationship between the CA activity and diabetes status may also be partially explained by the differing MG levels among the individuals in PD group of our study; however, MG levels were not determined in the current study.

## 5. Conclusions

The potential differences in environmental and lifestyle factors, as well as the fundamental differences in BMI and age between the Indian and Finnish study groups may explain the poor universal applicability of  $\delta_{\text{DOB}}^{18}\text{O}$  as a biomarker in prediabetes screening. In our study, fasting state  $\delta^{18}\text{O}$  was found to positively correlate with age, an anthropometric parameter showing, unintentionally, significant difference between the two study groups and being higher in PD group in comparison to NDC group. As for CA activity, some earlier studies have suggested that it reflects study subjects' BMI; in our study population of mainly middle-aged subjects, CA activity showed negative correlation with BMI. Thus, it seems that the application of  $\delta_{\text{DOB}}^{18}\text{O}$  as a CA activity and prediabetes biomarker in individual patients requires careful consideration of the multidimensional interactions between several geographic, climatic, and demographic variables. However, it is also possible that the participants in the current study fulfilling the diagnostic criteria of PD had already taken some action to improve their PD state, and therefore the differences between study groups may have been mitigated. Furthermore, although  $\delta_{\text{DOB}}^{18}\text{O}$  and CA activity did not seem to have a role as independent risk predictors in clinical screening, we cannot exclude the possibility that  $\delta^{13}\text{C}$  measurement could have an added value as part of the noninvasive screening panel including other validated measurements

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